# APPENDIX A

## Identification and Enumeration of *Prymnesium parvum* cells, Version AEW-IDE 1.1

GREGORY M. SOUTHARD

Purpose and Scope:
This protocol outlines the accepted procedure for identification and enumeration of *P. parvum* in water samples.

Materials required:
Standard hemacytometer
Cover slip
Light microscope with scanning and high dry objectives
Pasteur pipette

Reagents/consumables:
Immersion oil
Lugol's solution
- 200 mL DI water
- 20 mL glacial acetic acid
- 20 g KI
- 10 g I

Procedure:

1. Carefully load the hemacytometer chamber using a Pasteur pipette at the loading groove, allowing capillary action to pull the water sample under the cover slip until chamber is full.
2. Using the scanning objective, observe for small, fast moving organisms. This is a tentative indication that *P. parvum* is present.
3. Switch to the 40X objective and look for *P. parvum* at 400X magnification. *P. parvum* will be identified by its small size (9-12 μm), two large chloroplasts (which may appear C-shape or saddle-shape), two long flagella, and one haptonema. All of the structures can be seen using 400X magnification, except the haptonema that can be visualized using the oil immersion lens (i.e., 1000X magnification). However, the oil immersion lens cannot be used with the hemacytometer.
4. Count the number of *P. parvum* cells in each of the five large squares of the hemacytometer grid, in each of the chambers (a total of 10 counts). The average number of cells per chamber (5 large squares) is the total cell count. For statistical accuracy, the total cell counts for each chamber should be within 10% of each other. If there is greater than 50 cells per large square, dilute the water sample to minimize time.
5. Total cells per mL is determined as follows:

$$\text{Total cells/mL} = (\text{total cell count}/5) \times (1/\text{dilution}) \times 10^4$$

Counting cell graphic (from Burleson, F. G., T. M. Chambers, and D. L. Wiedbrauk. 1992). Virology – a laboratory manual. Academic Press, Inc. New York. 250 pp.

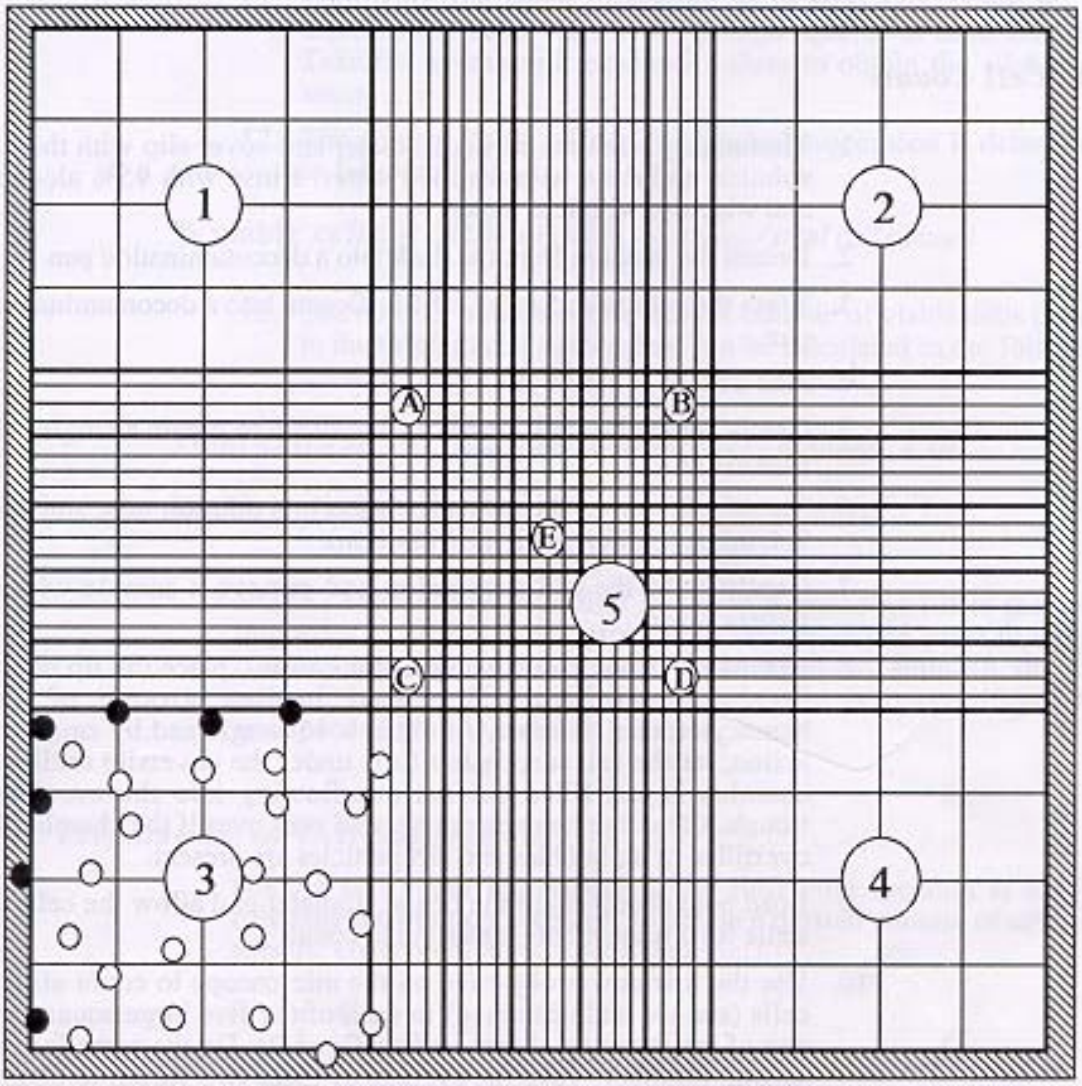


**Figure 5.2** Enlarged view of the hemacytometer counting chamber, as it would appear through the low power of the microscope. The cells in the five large squares (1-5) are counted and this number is divided by 5 to obtain the average number of cells per large square. This value is multiplied by $10^4$ and by the reciprocal of the dilution factor to obtain the number of cells/ml of the original cell suspension. Square #3 has a sample cell count. The open circles would be counted, whereas the dark circles indicate cells that would not be counted since they touch the top and left lines of that square.

Red blood cell counts are performed using the high dry objective of the microscope and counting the cells in 5 of the small squares (A,B,C,D, and E) of square #5. The cells/ml in the original suspension is calculated by multiplying the total number of cells in the 5 small squares by 5 x $10^4$, and the reciprocal of the dilution factor.

# APPENDIX B

## Standard Bioassay of *Prymnesium parvum* Toxin, Version AEW-ITU 1.2*[1]

GREGORY M. SOUTHARD

Purpose and Scope: This test should be used when *Prymnesium parvum* is detected in hatchery water. The purpose is to evaluate the concentration of prymnesin toxin in ichthyotoxic units ( ITU*) in hatchery water to determine the need for algaecide treatment.

*ITU (ichthyotoxic unit) = 1/25$^{th}$ the lethal dose of prymnesin ichthyotoxin/mL for fish.

Equipment required:
Glass/metal aquarium (ca. 10 L)
with aquarium heater (a water bath may be substituted if available)
Thermometer
5 beakers (100 mL-1L)
pH meter
Graduated cylinder
Pipettes (mL and μL)

Reagents/consumables:
Distilled water
Pond water (test water)
Cofactor solution (see below)
12 test fish

Cofactor Solution Preparation:

1. Cofactor: 0.003 M DADPA /0.02 M tris buffer in distilled water, pH = 9.0.
   To 491.15 mL of distilled water in a dark storage container, add 9.85 mL of DADPA (= 0.15 M) and 60.5 g tris (= 1.0 M)
2. Store in the dark at 4°C.

(Note: 1 mL cofactor solution per 50 mL water is needed for each of four test beakers. DADPA = 3,3'-iminobispropylamine, Sigma cat # I 7006, supplied as 1.772-M solution.)

Procedure:

1) Fill the aquarium or water bath with enough water to incubate but not float the beakers.
2) Maintain temperature at 28°C ± 1.
3) Five beakers are used for each test. Size beakers according to size of test fish. (Note: Fish lengths and beaker sizes are guidelines and may be adjusted as needed.)
4) Add 1 mL of prepared cofactor per 50-mL total volume in each of the 4 beakers. 'Clean' water used for dilutions and controls is from *P. parvum*-free source (See Table 1 below).
5) Add 4 test fish to each beaker.
6) Observe for mortality and maintain constant temperature in aquarium.
7) Results are noted after 2 hours.

[1] Adapted from the Central Fish Health Lab, Nir David, Israel.

Interpretation:

1) Record mortality in each beaker (see Table 1) after 2 hours.

a) Mortality in beaker #2 (undiluted pond water + cofactor) indicates the presence of at least 1 ITU/mL, which is $1/25^{th}$ the lethal dose to fish in ponds. Toxicity is considered to be low. Treatment is not required, but continued monitoring is advised.

b) Mortality in beakers #2 and #3 (1/5 dilution + cofactor) indicates 5 ITU/mL, one-fifth the dangerous level in the ponds, which indicates the need for immediate treatment of the ponds. Toxicity is considered to be moderate.

c) Mortality in beakers # 2, 3 and #1 (undiluted pond water) indicates a high level of toxicity (25 ITU/mL or greater). The pond is toxic and fish should be displaying signs of prymnesin toxicity.

d) Partial mortality can occur in a beaker, which would indicate a level of ichthyotoxicity between those parameters confined by the dilutions and cofactor used in the bioassay. For example, there can be complete mortality in beaker #2, but only partial (e.g. 1 of 3 test fish) in beaker #3, which would indicate an ITU level between 1 and 5 ITU/mL. The same phenomenon can occur at higher toxin levels, with complete mortality occurring in beakers #2 and #3, or with partial mortality in beaker #1, indicating an ITU level between 5 and 25 ITU/mL. Partial mortality cannot occur in two of the beakers and if does, some other lethal mechanism is likely the cause.

e) Mortality in either control beaker suggests some other lethal mechanism. If fish die in one of the control beakers, the test should be repeated.

TABLE 1.—Suggested beaker size and experimental conditions to test for the presence of ichthyotoxin (from *Prymnesium parvum*) in hatchery water. If more than one pond is tested at a time, beakers 1 and 2 must be repeated for each pond, but only one set of controls (beakers #4 and 5) is required.

| Fish length (mm) | Beaker size (mL) | Pond Water (mL) | Dilution water (mL)* | Cofactor (mL) | Dilution |
|---|---|---|---|---|---|
| ≤ 50 | 100 | 50 | 0 | 0 | 1:1 |
| | | 49 | 0 | 1 | 1:1 |
| | | 10 | 39 | 1 | 1:5 |
| | | control | 50 | 0 | |
| | | control + cofactor | 49 | 1 | |
| 50 < length ≤ 75 | 400 | 200 | 0 | 0 | 1:1 |
| | | 196 | 0 | 4 | 1:1 |
| | | 40 | 156 | 4 | 1:5 |
| | | control | 400 | 0 | |
| | | control + cofactor | 396 | 4 | |
| > 75 | 800 | 600 | 0 | 0 | 1:1 |
| | | 588 | 0 | 12 | 1:1 |
| | | 120 | 468 | 12 | 1:5 |
| | | control | 600 | 0 | |
| | | control + cofactor | 588 | 12 | |

* Dilution water must be free of *Prymnesium parvum* cells and toxin (e.g., conditioned tap water).

# APPENDIX C

Recommended treatments for *Prymnesium parvum* blooms using liquid ammonia, ammonium sulfate, or copper sulfate as related to temperature and pH (after the Central Fish Health Lab (Israel) recommendations).

| Temperature (ºC) | pH | Liquid ammonia (mg/L) | Ammonium sulfate (mg/L) | Copper sulfate (mg/L) |
|---|---|---|---|---|
| > 20 | > 9.0 | | 10-12 | |
| | 8.6-9.0 | 10-12 | 15 | |
| | < 8.6 | 12-13 | 15-17 | |
| 18-20 | > 9.0 | 10-12 | 15 | 2 |
| | 8.6-9.0 | 12-13 | 20 | 2 |
| | < 8.6 | 13 | 25 | |
| 12-18 | > 9.0 | 13 | 25 | 2-3 |
| | 8.6-9.0 | 13 | | 2-3 |
| | < 8.6 | | | 2-3 |

Note: The toxicity of copper sulfate increases as water alkalinity decreases and a more appropriate treatment concentration may be determined as follows:

Treatment rate (mg/L) = alkalinity as $CaCO_3$ ÷ 100.

# APPENDIX D

## Copper − Bathocuproine Method, Version AEW-COP 1.1

STEVEN HAMBY

**Purpose and Scope:** This protocol describes methods used for measuring copper at concentrations of 0.02 to 0.5 mg/L. Color and turbidity of the sample can affect the accuracy of the method.

**Materials Required:**

Equipment

Class A 100-mL or 50-mL glassware
(volumetric or graduated cylinder)
125-mL flask
Spectrophotometer, 1-cm path length
Hot plate

Reagents/consumables:

Deionized (D.I.) water that has been distilled in a glass still
1 +1 Hydrochloric acid (Conc. HCl diluted 1:1 v:v with water)
Hydroxylamine hydrochloride solution.
Sodium citrate solution
Disodium bathocuproine disulfonate solution
Stock copper solution (20 mg/L) for Cu standards:

- 10 mg/L intermediate
- 0.05 mg/L
- 0.1 mg/L
- 0.5 mg/L
- 0.2 mg/L

**Procedure:**

A. Prior to use, wash all glassware with concentrated HCl then rinse with copper-free water. Note: Many domestic waters have copper in detectable concentrations due to leaching from copper pipes.
B. Turn spectrophotometer on, set absorbance to 484 nm, and allow the unit to warm up.
C. Pour 50 mL of the blank (reagent water), standard, or sample into a separate 125-mL flask.
D. To each flask add, with mixing after each addition, the following: 1 mL (1 + 1) HCl, 5 mL $NH_2OH \cdot HCL$ solution, 5 mL sodium citrate solution, 5 mL disodium bathocuproine disulfonate solution.
E. After all flasks have been mixed, pour an aliquot into a cuvet, place it in the spectrophotometer, and record the absorbance. The following order is suggested: blank, 0.05 mg/L, 0.1 mg/L, 1 mg/L, samples, and the 0.5-mg/L continuing calibration verification standard.

**Calculations:**

Use the absorbencies from each standard to plot readings on a calibration curve to determine the amount of copper present.

**Chemical recipes:**

A. Hydroxylamine hydrochloride solution: 50g $NH_2OH \cdot HCL$ in 450 mL water.
B. Sodium citrate solution: dissolve 300 g $Na_3C_6H_5O_7 \cdot 2H_2O$ and make up to 1 L with water.
C. Disodium bathocuproine disulfonate solution: dissolve 1 g $C_{12}H_4N_2(CH_3)_2(C_6H_4)_2(SO_3Na)_2$ in water and make up 1 L.
D. Stock copper solution (20 mg/L): into a 250 mL flask, add 10 mL reagent water, 20 mg copper wire, and 5 mL conc. $HNO_3$. As reaction slows, warm to completely dissolve the copper, then boil to expel nitrogen oxides. Cool and add 50 mL reagent water, carefully transfer to a 1-L volumetric flask, and fill to 1 L.
E. Cu standards:

10 mg/L intermediate (50 mL of 20 mg/L to 100 mL) or (25 mL of 20 mg/L to 50 mL)
0.5 mg/L (5 mL of 10 mg/L to 100 mL)
0.1 mg/L (1 mL of 10 mg/L to 100 mL)
0.05 mg/L (5 mL of 1 mg/L to 100 mL)
0.2-mg/L continuing calibration verification standard (1 mL of 20 mg/L to 100 mL)

**Quality Control:**

A. A laboratory duplicate sample should be tested along with the original sample. An analytical precision control chart should be kept to monitor these results.
B. Percent recovery of the spike standard can be calculate as follows:

$$\% \text{ Recovery} = \frac{(Cs - Csx) \times 100}{Ca}$$

Where Cs = result of spiked sample
Csx = average of the original and duplicate sample
Ca = value of the spike added

**Reference:**

Standard Methods for the Examination of Water and Wastewater 3500-Cu E, 18th edition.

**NOTE**: This method measures the free copper. To measure the chelated form perform the preliminary digestion described in Standard Methods 3500-Cu Neocuproine Method 4b then adjust pH to 4-5.